



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C07K 7/10, 7/34, A61K 37/02 A61K 37/28	A1	(11) International Publication Number: WO 87/ 06941 (43) International Publication Date: 19 November 1987 (19.11.87)
(21) International Application Number: PCT/US87/01005 (22) International Filing Date: 5 May 1987 (05.05.87) (31) Priority Application Number: 859,928 (32) Priority Date: 5 May 1986 (05.05.86) (33) Priority Country: US (71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; Fruit Street (Bar-3), Boston, MA 02114 (US). (72) Inventor: HABENER, Joel ; 217 Plymouth Road, Newton Highlands, MA 02161 (US). (74) Agents: GOLDSTEIN, Jorge, A. et al.; Saidman, Sterne, Kessler & Goldstein, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US).		(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: INSULINOTROPIC HORMONE (57) Abstract A fragment of glucagon-like peptide I (GLP-1) has been found to be an insulinotropic hormone. This insulinotropic hormone comprises amino acid residues 7-37 of GLP-1. The insulinotropic hormone is useful as a potential therapy for <i>Diabetes Mellitus</i> .		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

-1-

INSULINOTROPIC HORMONE**BACKGROUND OF THE INVENTION****Field of the Invention**

. This invention is directed to the discovery that certain peptide fragments of the prehormone, proglucagon, possess hormonal activities and can be used to stimulate the synthesis and secretion of the hormone, insulin. These peptide fragments are useful in therapy for the disease Diabetes mellitus.

Description of the Background Art

The endocrine secretions of the pancreatic islets are under complex control not only by blood-borne metabolites (glucose, amino acids, catecholemines, etc.), but also by local paracrine influences. The major pancreatic islet hormones (glucagon, insulin and somatostatin) interact amongst their specific cell types (A, B, and D cells, respectively) to modulate secretory responses mediated by the metabolites. Although insulin secretion is predominantly controlled by blood levels of glucose, glucagon and somatostatin stimulate and inhibit glucose-mediated insulin secretory responses, respectively. In addition to the pro-

-2-

posed interislet paracrine regulation of insulin secretion, there is evidence to support the existence of insulinotropic factors in the intestine. This concept originates from the observations that glucose taken orally is a much more potent stimulant of insulin secretion than is a comparable amount of glucose given intravenously.

The human hormone, glucagon, is a 29-amino acid peptide hormone produced in the A-cells of the pancreas. The hormone belongs to a multi-gene family of structurally related peptides that include secretin, gastric inhibitory peptide, vasoactive intestinal peptide and glicentin. These peptides variously regulate carbohydrate metabolism, gastrointestinal mobility and secretory processing. The principal recognized actions of pancreatic glucagon, however, are to promote glycogenolysis and gluconeogenesis, resulting in an elevation of blood sugar levels. In this regard, the actions of glucagon are counterregulatory to those of insulin and may contribute to the hyperglycemia that accompanies Diabetes mellitus (Lund, P. K. et al., Proc. Natl. Acad. Sci., USA, 79: 345-349 (1982)).

Glucagon has been found to be capable of binding to specific receptors which lie on the surface of insulin producing cells. Glucagon, when bound to these receptors, stimulates the rapid synthesis of cAMP, by these cells. cAMP, in turn, has been found to stimulate insulin expression (Korman, L.Y. et al., Diabetes, 34:717-722 (1985)). Insulin acts to inhibit glucagon synthesis (Review of Medical Physiology, Ganong, W.F., 1979 Lange Publications, Los Altos, California (p. 273)). Thus the expression of glucagon is carefully

-3-

regulated by insulin, and ultimately by the serum glucose level.

The glucagon gene is initially translated from a 630 base pair precursor to form the polypeptide, preproglucagon (Lund et al. (1982)). This polypeptide is subsequently processed to form proglucagon. Patzelt, C. et al., Nature, 282: 260-266 (1979), demonstrated that proglucagon was subsequently cleaved into glucagon and a second polypeptide. Subsequent work by Lund, P. K. et al., Lopez L. C. et al., and Bell, G. I. et al., (Nature) 302:716-718 (1983) demonstrated that the proglucagon molecule was cleaved immediately after lysine-arginine dipeptide residues. Studies of proglucagon produced by channel catfish (Ictalurus punctata) indicated that glucagon from this animal was also proteolytically cleaved after adjacent lysine-arginine and arginine-arginine dipeptide residues (Andrews, P. C. et al., J. Biol. Chem., 260: 3910-3914 (1985)). Lopez, L. C. et al., (Proc. Natl. Acad. Sci. USA 80:5485-5489 (1983)), and Bell, G. I. et al., discovered the mammalian proglucagon was cleaved at lysine-arginine or arginine arginine dipeptides, and demonstrated that the proglucagon molecule contained three discrete and highly homologous peptide molecules which were designated glucagon, glucagon-like protein 1 (GLP-1) and glucagon-like protein 2 (GLP-2). Lopez et al. concluded that glucagon-like protein 1 was 37 amino acid residues long and that glucagon-like peptide 2 was 34 amino acid residues long. Analogous studies on the structure of rat preproglucagon revealed a similar pattern of proteolytic cleavage between adjacent lysine-arginine or arginine-arginine dipep-

-4-

tide residues, resulting in the formation of glucagon, GLP-1 and GLP-2 (Heinrich, G. et al., Endocrinol., 115: 2176-2181 (1984)). Human rat, bovine, and hamster sequences of GLP-1 have been found to be identical (Ghiglione, M. et al., Diabetologia, 27:599-600 (1984)).

The conclusion reached by Lopez et al. regarding the size of GLP-1 was confirmed by the work of Uttenthal, L.O. et al. (J. Clin. Endocrinol. Metabol., 61: 472-479 (1985)). Uttenthal et al. examined the molecular forms of GLP-1 which were present in the human pancreas. Their research shows that GLP-1 and GLP-2 are present in the pancreas as 37 amino acid and 34 amino acid peptides, respectively.

The similarity between GLP-1 and glucagon suggested to early investigators that GLP-1 might have biological activity. Although some investigators found that GLP-1 could induce rat brain cells to synthesize CAMP (Hoosein, N.M. et al., Febs Lett. 178:83-86 (1984)), other investigators failed to identify any physiological role for GLP-1 (Lopez, L. C. et al.). The failure to identify any physiological role for GLP-1 caused some investigators to question whether GLP-1 was in fact a hormone and whether the relatedness between glucagon and GLP-1 might be artifactual (Ghiglione, M. et al.).

Thus, in conclusion, the prior art reveals an awareness of the processing of a glucagon hormone precursor into a set of peptides sharing extensive homology. It has been widely assumed by those of skill in the art that these highly related glucagon-like peptides would have a biological activity. Nevertheless, extensive investigations designed to elucidate the

-5-

biological effects of these molecules had been unsuccessful.

SUMMARY OF THE INVENTION

The hormone glucagon is known to be synthesized as a high molecular weight precursor molecule which is subsequently proteolytically cleaved into three peptides: glucagon, glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2). GLP-1 has 37 amino acids in its unprocessed form. This invention discloses that the unprocessed GLP-1 is naturally converted to a 31 amino acid long peptide (7-37 peptide) having amino acids 7-37 of GLP-1. This processing occurs in the pancreas and the intestine. The 7-37 peptide is an insulinotropic hormone which had not previously been described. The hormone's activity appears to be specific for the pancreatic beta cells where it appears to induce the biosynthesis of insulin. The unprocessed GLP-1 peptide is essentially unable to mediate the induction of insulin biosynthesis. The insulinotropic hormone is useful in the study of the pathogenesis of maturity onset diabetes mellitus, a condition in which the dynamics of insulin secretion are abnormal. Moreover, the insulinotropic hormone is useful in therapy for this disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA structure and corresponding amino acid sequence of human, rat and hamster preproglucagons. The preproglucagon precursor is proteolytically cleaved at sites indicated by circles.

-6-

Figure 2 shows the effect of GLP-1 peptides on insulin mRNA levels in rat insulinoma cells.

Figure 3 shows the effects of GLP-1 peptides on angiotensinogen mRNA levels in rat insulinoma cells.

Figure 4 shows the effects of GLP-1 peptides on actin mRNA levels in rat insulinoma cells.

Figure 5 shows the effect of GLP-1 (1-37) on prolactin mRNA levels in GH4 cells.

Figure 6 shows the effects of GLP-1 (1-37) on ACTH mRNA levels in AtT-20 cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Peptide moieties (fragments) chosen from the determined amino acid sequence of human GLP-1 constitute the starting point in the development comprising the present invention. The amino acid sequence for GLP-1 has been reported by several researchers (Lopez, L. C. et al. (1983); Bell, G. I. et al., (Nature) 302:716-718 (1983); Heinrich, G. et al. (1984); Ghiglion, M. et al. (1984)). The structure of the preproglucagon gene and its corresponding amino acid sequence is shown in Figure 1. This figure further displays the proteolytic processing of the precursor gene product into glucagon and the two glucagon-like peptides. As used herein, the notation GLP-1 (1-37) refers to a GLP-1 polypeptide having all amino acids from 1 (N-terminus) through 37 (C-terminus). Similarly, GLP-1 (7-37) refers to a GLP-1 polypeptide having all amino acids from 7 (N-terminus) through 37 (C-terminus).

In one embodiment, the peptide fragments are synthesized by the well-known solid phase peptide syn-

-7-

thesis described by Merrifield, J. M., Chem. Soc., 85: 2149 (1962), and Stewart and Young, Solid Phase Peptide Synthesis (Freeman, San Francisco, 1969), pages 27-66, which are incorporated by reference herein. However, it is also possible to obtain fragments of the proglucagon polypeptide or of GLP-1 by fragmenting the naturally-occurring amino acid sequence, using, for example, a proteolytic enzyme. Further, it is possible to obtain the desired fragments of the proglucagon peptide or of GLP-1 through the use of recombinant DNA technology, as disclosed by Maniatis, T. et al., Molecular Biology: A Laboratory Manual, Cold Spring Harbor, NY 1982, which is hereby incorporated by reference.

The invention pertains to a peptide fragment which is insulinotropic and is derivable from a naturally-occurring amino acid sequence.

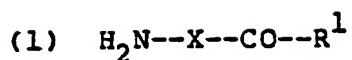
The invention comprises a peptide fragment having the following amino acid sequence:

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-
Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-
Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-
Lys-Gly-Arg-Gly

and functional derivatives thereof, these fragments and functional derivatives being substantially free of natural contaminants and having insulinotropic activity.

Of particular interest are peptides of the following formula:

-8-



wherein R^1 is OH, OM or $-\text{NR}^2\text{R}^3$;

M is a pharmaceutically acceptable cation or a lower ($\text{C}_1\text{--C}_6$) branched or unbranched alkyl group; R^2 and R^3 are the same or different and selected from the group consisting of hydrogen and a lower ($\text{C}_1\text{--C}_6$) branched or unbranched alkyl group; and

X is the amino acid sequence or peptide fragment described above;

(2) The acid addition salts thereof; and

(3) The protected or partially protected derivatives thereof.

The invention further pertains to a method for enhancing the expression of insulin which comprises:

providing to a mammalian pancreatic B-type islet cell an effective amount of the insulinotropic peptides disclosed above.

Included within the scope of the present invention are those amino acid sequences in the above peptides which are capable of functioning as insulinotropic hormones. Included as well are the use of additional amino acid residues added to enhance coupling to carrier protein or amino acid residues added to enhance the insulinotropic effect. A material is said to be "substantially free of natural contaminants" if it has been substantially purified from materials with which it is normally and naturally found. Examples of natural contaminants with which GLP-1 (7-37) might be

-9-

associated are: other peptides, carbohydrates, glycosylated peptides, lipids, membrane, etc. A material is also said to be substantially free of natural contaminants if these contaminants are substantially absent from a sample of the material.

The interchangeable terms "peptide fragment" and "peptide moiety" are meant to include both synthetic and naturally-occurring amino acid sequences derivable from a naturally occurring amino acid sequence.

A peptide is said to be "derivable from a naturally-occurring amino acid sequence" if it can be obtained by fragmenting a naturally-occurring sequence, or if it can be synthesized based upon a knowledge of the sequence of the naturally occurring amino acid sequence or of the genetic material (DNA or RNA) which encodes this sequence.

The invention further pertains to polypeptides that, in addition to the chosen sequence, may contain or lack one or more amino acids that may not be present in the naturally-occurring sequence wherein such polypeptides are functionally similar to the chosen polypeptide. Such polypeptides for the present invention, are termed "functional derivatives," provided that they demonstrate insulintropic activity which is substantially similar to that of GLP-1 (7-37).

An "insulintropic activity" relates to the ability of a substance to stimulate, or cause the stimulation of, the synthesis or expression of the hormone insulin.

As is known in the art, the amino acid residues may be in their protected or unprotected form, using appropriate amino or carboxyl protecting groups. Use-

-10-

ful cations are alkali or alkaline earth metallic cations (i.e., Na, K, Li, $1/2\text{Ca}$, $1/2\text{Ba}$, etc.) or amine cations (i.e., tetraalkylammonium, trialkylammonium, where alkyl can be $\text{C}_1\text{-C}_{12}$).

The variable length peptides may be in the form of the free amines (on the N-terminus), or acid-addition salts thereof. Common acid addition salts are hydrohalic acid salts, i.e., HBr, HI, or more preferably, HCl.

The insulinotropic property of a compound may be determined by providing that compound to animal cells, or injecting that compound into animals and monitoring the release of immunoreactive insulin (IRI) into the media or circulatory system of the animal, respectively. The presence of IRI is detected through the use of a radioimmunoassay which can specifically detect insulin. Although any radioimmunoassay capable of detecting the presence of IRI may be employed, it is preferable to use a modification of the assay method of Albano, J.D.M., et al. (Acta Endocrinol. 70: 487-509 (1972)). In this modification a phosphate/albumin buffer with a pH of 7.4 was employed. The incubation was prepared with the consecutive condition of 500 ul of phosphate buffer, 50 ul of perfusate sample or rat insulin standard in perfusate, 100 ul of anti-insulin antiserum (Wellcome Laboratories; 1:40,000 dilution), and 100 ul of [^{125}I] insulin, giving a total volume of 750 ul in a 10 X 75-mm disposable glass tube. After incubation for 2-3 days at 4°C , free insulin was separated from antibody-bound insulin by charcoal separation. The assay sensitivity was 1-2 uU/ml. In order to measure the release of IRI

-11-

into the cell culture medium of cells grown in tissue culture, one preferably incorporates radioactive label into proinsulin. Although any radioactive label capable of labelling a polypeptide can be used, it is preferable to use ^3H leucine in order to obtain labelled proinsulin. Labelling can be done for any period of time sufficient to permit the formation of a detectably labelled pool of proinsulin molecules; however, it is preferable to incubate cells in the presence of radioactive label for a 60 minute time period. Although any cell line capable of expressing insulin can be used for determining whether a compound has an insulintropic effect, it is preferable to use rat insulinoma cells, and especially RIN - 38 rat insulinoma cells. Such cells can be grown in any suitable medium; however, it is preferable to use DME medium containing 0.1% BSA and 25 mM glucose.

The insulintropic property of a compound may also be determined by pancreatic infusion. The in situ isolated perfused rat pancreas preparation was a modification of the method of Penhos, J. C. et al., (Diabetes, 18:733-738 (1969)). Fasted male Charles River strain albino rats, weighing 350-600 g, were anesthetized with an intraperitoneal injection of Amytal Sodium (Eli Lilly and Co.; 160 mg/kg). Renal, adrenal, gastric, and lower colonic blood vessels are ligated. The entire intestine was resected except for about four cm of duodenum and the descending colon and rectum. Therefore, only a small part of the intestine was perfused, thus minimizing possible interference by enteric substances with glucagon-like immunoreactivity. The perfusate was a modified Krebs-Ringer bicar-

-12-

bonate buffer with 4% dextran T70 and 0.2% bovine serum albumin (fraction V), and was bubbled with 95% O₂ and 5% CO₂. A nonpulsatile flow, 4-channel roller bearing pump (Buchler polystatic, Buchler Instruments Division, Nuclear-Chicago Corp.) was used, and a switch from one perfusate source to another was accomplished by switching a 3-way stopcock. The manner in which perfusion was performed, monitored, and analyzed followed the method of Weir, G. C. et al. (J. Clin. Investigat. 54: 1403-1412 (1974)), which is hereby incorporated by reference.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby GLP-1 (7-37) or its functional derivatives are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g. human serum albumin, are described for example in Remington's Pharmaceutical Sciences (16th Ed. A. Oslo Ed. Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the GLP-1 (7-37), or its functional derivatives, together with a suitable amount of carrier vehicle.

Compositions containing GLP-1 (7-37) or its functional derivatives may be administered intravenously, intramuscularly, or subcutaneously at dosages in the range of from about 1 pg/kg to 1,000 ug/kg body weight, although a lower or higher dosage may be administered. The required dosage will depend upon the

-13-

severity of the condition of the patient and upon such criteria as the patient's height, weight, sex, age, and medical history.

For the purpose of parenteral administration, compositions containing GLP-1 (7-37) are dissolved in distilled water and the pH-value is adjusted to about 6 to 8. In order to facilitate the lyophilization process resulting in a suitable product, lactose could be added to the solution. The solution is then filter sterilized, introduced into vials, and lyophilized. The concentration of GLP-1 (7-37) in these compositions may vary from 10^{-12} M to 10^{-5} M.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to complex or adsorb GLP-1 (7-37) or its functional derivatives. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, and protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate GLP-1 (7-37) into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating GLP-1 (7-37) into these polymeric particles, it is possible to entrap GLP-1 (7-37) in microcapsules prepared, for example,

-14-

by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly (methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such teachings are disclosed in Remington's Pharmaceutical Sciences (1980).

SPECIFIC EXAMPLES

EXAMPLE 1

Rat insulinoma cells of cell line RIN-38 were derived from a continuous islet cell line, RIN-r, which was established from a transplantable rat islet cell tumor (Gazdar, A. F. et al., Proc. Nat'l Acad. Sci. U.S.A. 77: 3519-3523 (1980)). The cells were maintained in DMEM (Gibco) at a glucose concentration of 4,500 mg/L, and supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml of penicillin and 100 ug/ml of streptomycin. Incubations were carried out at 37°C in 95% air: 5% CO₂. Cells grown in the above manner were washed and resuspended in DMEM (Gibco) containing 0.1% bovine serum albumin and 25 mM glucose. Cells were incubated with varying concentrations of GLP-1 (1-37), GLP-1 (7-37) or GLP-1 (1-36 des-gly-arg amide) for six hours, following which the effects of these agents on insulin mRNA expression were determined. Cellular RNA was analyzed for insulin specific mRNA as follows: cellular RNA was ex-

-15-

tracted from solid tumors and cells by homogenization in guanadine thiocyanate and sedimentation through a cesium chloride cushion. Poly A⁺ RNA was isolated by oligo dT cellulose chromatography (Aviv, H. et al., Proc. Nat'l Acad. Sci. U.S.A. 69: 1408-1412 (1972)). 20 ug of total RNA from each sample was fractionated by size on a 1.4% agarose gel after denaturation in glyoxal, followed by electrotransfer to a nylon membrane (Nytran; Schleicher and Schuell). Blotted membranes were baked for two hours at 80°C under vacuum, prehybridized in 1M NaCl / 1% SDS/ 10% Dextran sulfate at 50°C overnight and hybridized at the same temperature for 24 h after addition of the labelled probes ($3-5 \times 10^5$ cpm/ml); they were then washed at 55°C twice in 1 X SSC (0.15 M NaCl / 0.015M Na citrate) / 1% SDS, and exposed to X-ray film for varying times at -70°C with an intensifying screen. In all cases the concentration of peptides was 10^{-7} M.

The result of this experiment is shown in Figure 2. Lanes 1-3 (control cells), 4-6 (GLP-1 (1-37)), 7-9 GLP-1 (7-37), 10-12 (GLP-1(1-36 des- gly arg -amide) shows the amount of insulin specific mRNA produced. Triplicate experimental results are presented for each peptide.

Using a microdensitometer the relative amounts of insulin specific mRNA were determined. This experiment revealed that, at equal peptide concentrations, GLP-1 (7-37) stimulated insulin gene expression to more than 3 times the level found in control (untreated) cells.

-16-

EXAMPLE 2:

Rat insulinoma cells of cell line RIN-38 were grown in DME medium as described in Example 1. After incubation with 10^{-7} M GLP-1 (1-37, GLP-1 (7-37) and GLP-1 (1-36), the concentrations of insulin in the cell culture mediums were determined by radioimmunoassay (as described above). Insulin protein levels were determined after incubation for six hours. The results of this experiment are shown in Table 1.

TABLE 1

PEPTIDE ADDED	Insulin Produced (uUnits/ML)
None	2800
GIP-1 (1-37)	5000

EXAMPLE 3:

The pancreas of live rat was perfused with varying concentrations of GLP-1 (1-37) and GLP-1 (7-37) as described above. At one minute intervals, rat serum insulin levels in picograms/ml were determined by radioimmunoassay (as described above). The results of this experiment are shown in Table 2. Perfusions were done using peptide concentrations of 5×10^{-7} M, 5×10^{-8} M, and 5×10^{-10} M, 5×10^{-11} M and 5×10^{-12} M. Peptides were added after the zero minute serum value had been determined.

-17-

GLP-1 (1-37) was found to mediate a 3.4-fold increase in serum insulin concentrations when perfused into rat pancreas at a concentration of $5 \times 10^{-7} \text{M}$; at a concentration of $5 \times 10^{-8} \text{M}$ this peptide was capable of mediating only a 2-fold increase in serum insulin levels. At a concentration of $5 \times 10^{-10} \text{M}$ this peptide was found to mediate only a 20% increase in serum insulin levels.

GLP-1 (7-37) was found to be capable of stimulating a 132-fold increase in insulin levels when provided to rat pancreas at a concentration of $5 \times 10^{-7} \text{M}$. At a 10-fold lower concentration ($5 \times 10^{-8} \text{M}$) this peptide was capable of directing a 21-fold increase in the serum concentration of insulin. At a concentration of $5 \times 10^{-10} \text{M}$, GLP-1 (7-37) was found to be capable of mediating an increase in serum insulin levels (32-fold). Even at a concentration of $5 \times 10^{-11} \text{M}$, GLP-1 (7-37) delivered a 15-fold increase in insulin levels whereas GLP-1 (1-37) was without effect.

This experiment shows that GLP-1 (7-37) is more than 1,000-fold more potent than GLP-1 (1-37) in stimulating insulin expression in vivo. In addition, the GLP-1 peptides had no effects on the release of the peptide hormones glucagon and somatostatin in these same experiments. Thus, the stimulatory effects of GLP-1 are specific for the beta cells and do not act on pancreatic alpha or delta cells.

-18-

Table 2

Insulin Produced (picograms/ml) at
Peptide Concentration

	<u>Time</u> <u>(Minutes)</u>	<u>$5 \times 10^{-7} \text{ M}$</u>	<u>$5 \times 10^{-8} \text{ M}$</u>	<u>$5 \times 10^{-10} \text{ M}$</u>	<u>$5 \times 10^{-11} \text{ M}$</u>	<u>$5 \times 10^{-12} \text{ M}$</u>
GLP-1 (7-37)	0	50	925	205	160	50
	1	6600	20,700	7400	2400	50
	2	4700	10,500	1800	1700	50
	3	1700	4,000	760	1900	98
GLP-1 (1-37)	0	1400	3,000	500	340	50
	1	4700	6,000	600	180	50
	2	2900	2,000	640	230	160
	3	2200	2,000	430	340	50

-19-

EXAMPLE 4:

In order to determine whether glucagon-like proteins were capable of affecting cellular cAMP levels the effects of GLP-1 (7-37) and GLP-1 (1-37) on cAMP levels in RINS-38 insulinoma cells was determined. Cells were grown as described in Example 1, in 26 well culture dishes. Varying amounts of glucogon-like peptides were added to culture wells in triplicate. After permitting incubation for 10 minutes the total cell media was examined for cAMP, and the concentration of cAMP was determined. The results of this experiment are shown in Table 3. 20 ul from each culture well was assayed.

Table 3

Peptide Concentration	<u>pMOLES OF cAMP PRODUCED</u>	
	Expt I	Expt II
(M)		
0	140	91
10 ⁻⁶	400	170
10 ⁻⁷	370	120
10 ⁻⁸	494	160
10 ⁻⁹	515	100
10 ⁻¹⁰	253	90
10 ⁻¹¹	533	90

This experiment reveals that GLP-1 (7-37) was capable of stimulating cAMP levels even when present at a

-20-

concentration of 10^{-11} M. The increase in cAMP levels is an indication that GLP-1 (7-37) is capable of interacting with cellular receptors.

EXAMPLE 5

In order to demonstrate that the effects of GLP-1 (1-37), GLP-1 (1-36) and GLP-1 (7-37) were specific for insulin, and were not capable of inducing or provoking non-specific gene expression, the effect of these peptides on the levels of actin and angiotensinogen mRNAs were conducted. RIN-38 insulinoma cells were grown as described in Example 1 and incubated in the presence of GLP-1 (1-37), GLP-1 (7-37), or GLP-1 (1-36) des-Gly arg (Peninsula Laboratories). In all cases the concentration of peptides was 10^{-7} M. Incubations were for six hours. Messenger RNAs specific for insulin, actin, or angiotensinogen were identified by Northern hybridization as described in Example 1. The results of this experiment are shown in Figure 2 (insulin mRNA); Figure 3 (angiotensinogen mRNA); and Figure 4 (actin mRNA). mRNA levels were determined in arbitrary densitometric units obtained from scanning films of the RNA gels of Figures 2, 3, and 4. The mRNA levels are shown in Table 4.

-21-

TABLE 4

EFFECTS OF GLUCAGON-LIKE PEPTIDES ON
CELLULAR LEVELS OF mRNAs ENCODING
INSULIN, ACTIN AND ANGIOTENSINOGEN
IN RIN-38 INSULINOMA CELLS

<u>PEPTIDE*</u>	<u>MESSENGER RNAs</u>		
	<u>INSULIN</u>	<u>ACTIN</u>	<u>ANGIOTENSINOGEN</u>
GLP-I (7-37)	4.23±0.74	0.82±0.08	2.78±0.46
GLP-I (1-37)	1.87±0.56	0.91±0.02	2.25±0.20
GLP-I (1-36) des-Gly Arginineamide	2.78±0.80	0.88±0.03	2.56±0.22
Control (no peptide)	1.28±0.23	0.89±0.05	2.67±0.31

EXAMPLE 6

GLP-1 (1-37) was examined to determine whether it could induce the biosynthesis of hormones other than insulin. Thus, GLP-1 (1-37) (at a concentration of 10^{-7} M) was added to a rat islet glucagon-producing cell line and two pituitary cell lines (GH4 and AtT-20) which were capable of producing the hormones prolactin and ACTH, respectively, and the amount of hormone specific mRNA produced was determined after 24 hours as described in Example 1. Prolactin mRNA levels in GH4 pituitary cells after incubation with GLP-1 peptides is shown in Figure 5. ACTH mRNA levels in AtT-20 pituitary cells after incubation with GLP-1 peptides is shown in Figure 6. These experiments revealed that GLP-1 (1-37) did not have any detectable effect upon the amount of mRNA which encodes these peptide hormones.

-22-

EXAMPLE 7

The effect of GLP-1 (7-37) on the transcription of the insulin and actin genes in RIN-38 insulinoma cells was investigated. Gene transcription rates were determined by quantification of nascent glucagon and beta-actin RNA transcripts in nuclei from control and TPA treated cells. Nuclear RNA was hybridized to an excess of cloned specific DNA bound to nitrocellulose and the filters were washed as described by McKnight, G. S. et al, (J. Biol. Chem. 254:9050-9058 (1979)). Rat glucagon (Heinrich, G. et al, Endocrinology, 115: 1-6, (1984)) and, for control, chicken beta-actin cDNAs, provided by Dr. D. Cleveland, the Johns Hopkins University School of Medicine, Baltimore, Maryland, were used. Hybridization efficiency was controlled through the addition of the hybridization solution of [³H] UTP glucagon cRNA. Experiments were done in duplicate and values are expressed in ppm/kb of cDNA insert, corrected for efficiency of hybridization (40-50%). Cells were incubated with GLP-1 (7-37) at a concentration of 10^{-7} M for 4 hours. Nuclei were prepared from cells at 0, 1 and 4 hours and nascent insulin gene and actin gene transcripts were assessed by the nuclear run on assay (McKnight, G. S. et al). The results of this experiment are shown in Table 5. The experiment shows that GLP-1 (7-37) increases the rate of insulin gene transcription, but has no detectable effect upon the rate of actin gene expression.

-23-

TABLE 5

EFFECT OF GLUCAGON-LIKE PEPTIDE I (7-37)
ON TRANSCRIPTION OF THE INSULIN AND ACTIN GENES
IN RIN-38 INSULINOMA CELLS

<u>TIME (hrs)</u>	<u>INSULIN GENE</u>	<u>ACTIN GENE</u>
0	17.4	34.1
1	76.2	29.9
4	9.0	25.0

Having now fully described this invention, it will be apparent to one of ordinary skill in the art that the same may be carried out with minor modifications which do not effect the content or spirit thereof.

-24-

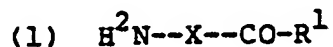
WHAT IS CLAIMED IS:

1. A peptide fragment which comprises the sequence:

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-
Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-
Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-
Lys-Gly-Arg-Gly

and functional derivatives thereof, said fragment and said functional derivatives being substantially free of natural contaminants and having insulinotropic activity.

2. A peptide having the formula:



wherein R^1 is OH, OM or $-\text{NR}^2\text{R}^3$;

M is a pharmaceutically acceptable cation or a lower branched or unbranched alkyl group;

R^3 and R^4 are the same or different and selected from the group consisting of hydrogen and a lower branched or unbranched alkyl group; and

X is the peptide fragment of claim 1;

- (2) the acid addition salts thereof; and
- (3) the protected or partially protected derivatives thereof.

-25-

3. The peptide fragment of claim 1 which is:

His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-
Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-
Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-
Lys-Gly-Arg-Gly

4. A method for enhancing the expression of insulin which comprises:

providing to a mammalian pancreatic B-type islet cell an effective amount of the insulinotropic peptide of claim 1.

5. A method for enhancing the expression of insulin which comprises:

providing to a mammalian pancreatic B-type islet cell an effective amount of the insulinotropic peptide of claim 2.

6. A method for enhancing the expression of insulin which comprises:

providing to a mammalian pancreatic B-type islet cell an effective amount of the insulinotropic peptide of claim 3.

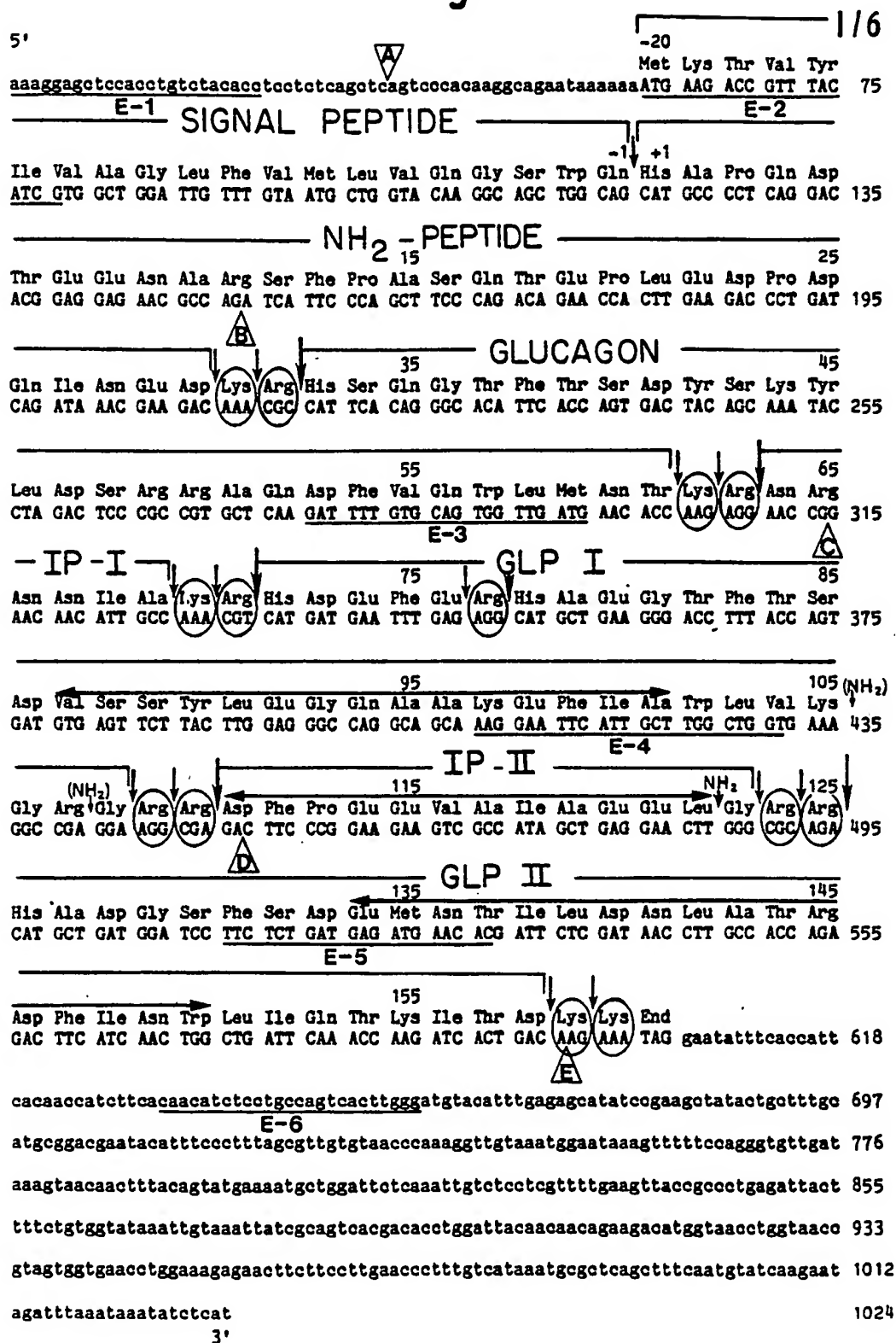
7. A pharmaceutical composition which comprises an effective amount of the peptide fragment of claim 1, in association with a suitable physiologically acceptable carrier, when used as an insulinotropic medicament.

-26-

8. A pharmaceutical composition which comprises an effective amount of the peptide fragment of claim 2, in association with a suitable physiologically acceptable carrier, when used as an insulintropic medicament.

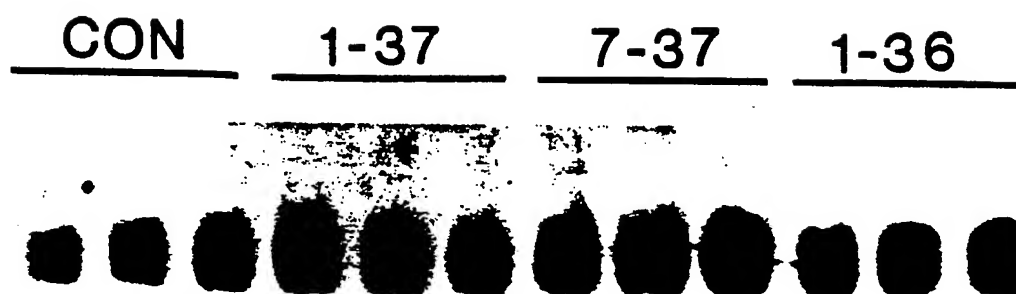
9. A pharmaceutical composition which comprises an effective amount of the peptide fragment of claim 3, in association with a suitable physiologically acceptable carrier, when used as an insulintropic medicament.

Fig.1



2/6

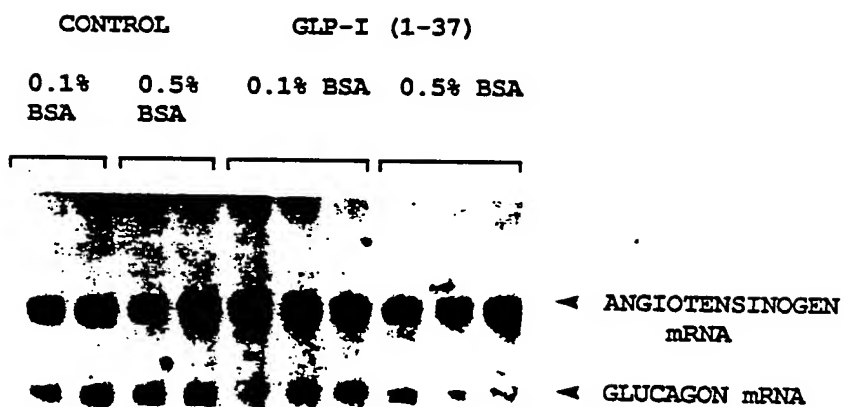
Fig.2



SUBSTITUTE SHEET

3 / 6

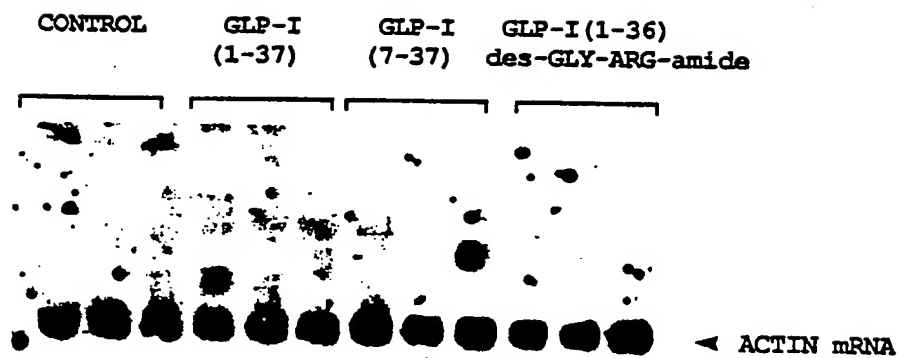
Fig. 3



SUBSTITUTE SHEET

4/6

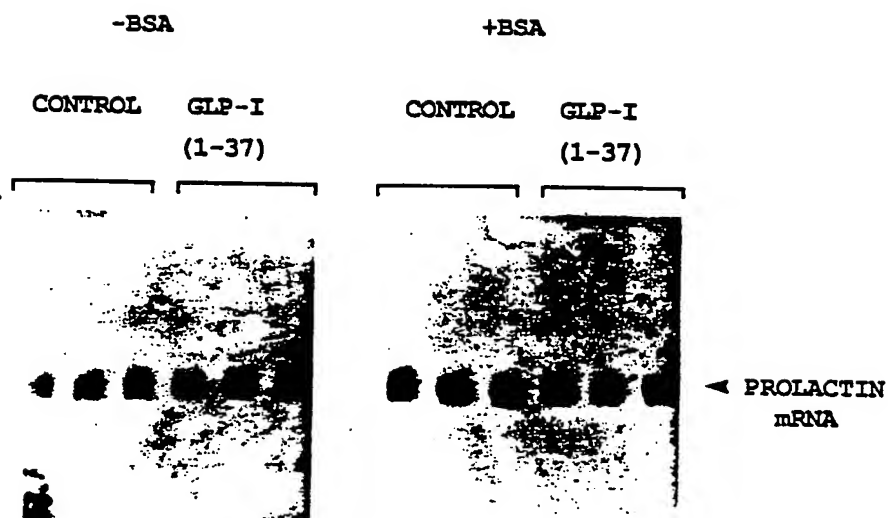
Fig.4



3183-1110-1111

5/6

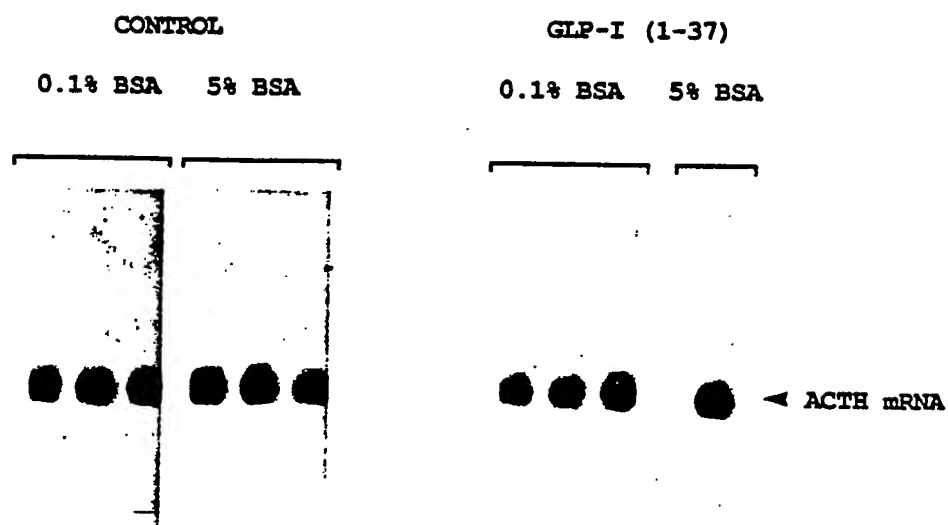
Fig. 5



SUBSTITUTE SHEET

6/6

Fig. 6



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No **PCT/US87/01005**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (4) C07K 7/10, 7/34; A61K 37/02, 37/28 US CL 530/324, 308; 514/12		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/324, 308; 514/12	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
BIOLOGICAL ABSTRACTS AND CHEMICAL ABSTRACTS ON LINE COMPUTER		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
P,X	J. CLIN. INVEST., VOL. 79 PAGES 616-619 PUBLISHED FEBRUARY 1987, (MOJSOV ET AL)	1-9
X Y	DIABETOLOGIA, VOL. 28 PAGES 704-707 PUBLISHED 1985 (SCHMIDT ET AL)	1-2,7-8 1-9
Y	DIABETOLOGIA, VOL. 27 PAGES 599-600, PUBLISHED 1984 (GHIGLIONE ET AL)	1-9
X Y	REGULATORY PEPTIDES VOL. 13 PAGE 101 ABS., PUBLISHED 1985 (GNIGLIONE ET AL)	1-2,7-8 1-9
A	ENDOCRINOL. VOL. 115 PAGES 2176-2181 PUBLISHED 1984 (HEINRICH ET AL)	1-9
A	NATURE VOL. 282 PAGES 260-266 PUBLISHED 15 NOVEMBER 1979 (PATZELT ET AL)	1-9
A	NATURE VOL. 289 PAGES 511-514 PUBLISHED 05 FEBRUARY 1981 (SHIELDS ET AL)	1-9
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹	Date of Mailing of this International Search Report ¹	
08 JUNE 1987	26 JUN 1987	
International Searching Authority ¹	Signature of Authorized Officer ¹⁹	
ISA/US	CHRISTINA CHAN <i>Christina Chan</i>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
A	PROC. NATL. ACAD. SCI. USA VOL. 79 PAGES 345-349 PUBLISHED JANUARY 1982 (LUND ET AL)	1-9
A	NATURE VOL. 302 PAGES 716-718 PUBLISHED 21 APRIL 1983 (BELL ET AL)	1-9
A	PROC. NATL. ACAD. SCI. USA VOL. 80 PAGES 5485-5489 PUBLISHED SEPTEMBER 1983 (LOPEZ ET AL)	1-9
A	J. BIOL. CHEM. VOL. 260 PAGES 3910-3914 PUBLISHED 10 APRIL 1985 (ANDREWS ET AL)	1-9
A	J. CLIN. END. METAB. VOL. 61 PAGES 472-479 PUBLISHED 1985 (UTTENTHAL ET AL)	1-9
A	NATURE VOL. 289 PAGES 514-516 PUBLISHED 05 FEBRUARY 1981 (MOODY ET AL)	1-9
A	FEBS LETTERS VOL. 187 PAGES 307-310 PUBLISHED AUGUST 1985 (YANAIHARA ET AL)	1-9
A	FEBS LETTERS VOL. 178 PAGES 83-86 PUBLISHED DECEMBER 1984 (HOSEIN ET AL)	1-9
A	DIABETES VOL. 34 PAGES 717-722 PUBLISHED 1985 (KORMAN ET AL)	1-9
A	EP, A, 008,2731, (JORGENSEN) 22 DECEMBER 1982	1-9
A	EP, A, 0044168 (LUNDT ET AL) 30 JUNE 1981	1-9